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**APPLICATION NUMBER: 60/541,632**

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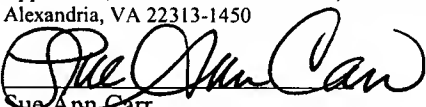
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**PROVISIONAL APPLICATION FOR PATENT  
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**Certificate Under 37 CFR 1.10**  
Date of Deposit: February 4, 2004  
I hereby certify that this correspondence is being deposited with the United States Postal Service as "Express Mail" service under 37 CFR 1.10 on the date indicated above addressed to Mail Stop Provisional Application, Commissioner for Patents, PO Box 1450, Alexandria, VA 22313-1450  
  
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This is a request for filing a Provisional Application for Patent under 37 CFR 1.53(c)

Inventor(s) and Residence(s) (city and either state or foreign country):

Last Name	First Name	Middle Initial	City	State or Country
Rekosh	David		Earlsville	Virginia
Hammarckjöld	Marie-Louise		Earlsville	Virginia

Title: **COMPOUNDS THAT INHIBIT HIV PARTICLE FORMATION FOR THE TREATMENT OF AIDS**

14 Sheets of specification.  
7 Sheets of drawings.

University of Virginia Patent Foundation claims small entity status as a nonprofit organization (37 CFR §§1.27(a)(3) and (c)). The Commissioner is hereby authorized to charge the Small Entity Fee of **\$80** to Deposit Account No. **50-0423**.

Please direct all communication relating to this application to:

John P. Breen, Esq.

Customer No. 34444

Patent Counsel

Telephone: (434) 243-6103

University of Virginia Patent Foundation

Fax: (434) 924-2493

1224 West Main Street, Suite 1-110

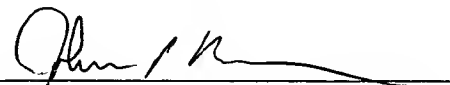
Charlottesville, VA 22903 U.S.A.

This invention was made by an agency of the United States Government or under a contract with an agency of the United States Government. The government has certain rights in the invention.

YES ☒ NO ☐ Grant No. R21 AI54213-01 and R21 AI54213-02

Dated: February 4, 2004

Respectfully submitted,

By:   
John P. Breen (Reg. No. 38,833)

## **Compounds that inhibit HIV particle formation for the treatment of AIDS**

### **US Government Rights**

This invention was made with United States Government support under Grant Nos. R21 AI54213-01, and R21 AI54213-02, awarded by National Institutes of Health. The United States Government has certain rights in the invention.

### **Background**

The present invention is directed to a safe quantitative *in vitro* high-throughput assay to screen library compounds for effects on Rev-dependent p24 production. Two cell lines derived from COS cells provide the means of determining whether a library compound has anti-Rev activity; 5BD.1 and 2A.22. These cell lines constitutively express HIV-like particles that contain the HIV core proteins as well as HIV envelope protein. The non-infectious virions created by these cells are secreted into the media, where a simple p24 ELISA can quantitatively determine virion production.

The Production of these virus-like particles is totally dependent on the expression of the Rev protein (for the 5BD.1 cell line), which is also made in these cell lines, or independent of Rev protein expression (the 2A.22 cell line). The cell lines are useful as packaging cells for HIV vectors, and also for drug screening using the HIV Rev protein as a target. The 5BD.1 and 5BA.1 cell lines described here are a considerable improvement over the previous B4.14 cell line, in that they express the HIV envelope protein, which the B4.14 cell lines does not and the cell also produces higher levels of p24.

The HIV virus particle consists of internal proteins that make up the viral core and two proteins that are part of the lipid envelope that surrounds the core. These proteins are expressed from precursor molecules called Pr55gag and Pr160gagpol for the core proteins and gp160 for the envelope proteins. Studies in our laboratory and elsewhere have demonstrated expression of these proteins normally requires co-expression of the HIV Rev protein. Without the Rev protein, the mRNAs encoding each of these proteins remains in the nucleus. In order for the Rev protein to work, it is also essential to have an element present in the RNA that binds to Rev. This element is called the RRE.

Using a vector that expressed Pr55gag and Pr160 and a vector that expressed Rev, we originally created the cell line called B4.14. This cell line was made at the State University of New York in Buffalo. B4.14 expressed HIV-like particles without the envelope protein. The creation of this cell line was described in the following publication: Srinivasakumar, N., Chazal, N., Helga-Maria, C., Prasad, S., Hammar skjöld, M.-L., and Rekosh, D. (1997) The effect of viral regulatory protein expression on gene delivery by human immunodeficiency virus type 1 vectors produced in stable packaging cell lines. *J. Virol* 71:5841-5848 .

At the University of Virginia, the B4.14 cell line was modified to also express the HIV envelope protein, by transfecting the cell line with a vector that expressed the protein. The resulting cell lines isolated are called 5BD.1 and 5BA.1. 5BA.1 is simply a different clone that was isolated at the same time as 5BD.1. There appears to be little difference in the properties of the two cell lines. One feature of these cell lines, relative to the parental B4.14, is that they express 2-4 times more p24 (HIV core) protein depending on the culture conditions.

Work in our laboratory led to the identification of a small RNA element from Mason-Pfizer Monkey Virus. When this element is present in the RNA that is expressed from a gene that normally requires Rev co-expression the need for Rev is overcome.

Our initial findings are described in detail in two patents and published paper:

“Purified retroviral constitutive transport enhancer elements that enhance nucleocytoplasmic transport of mRNA and methods of making and using the elements” US Patent # 5880276 Issued 3/9/99.

“A purified retroviral constitutive transport enhancer and its use to facilitate mRNA transport, and to produce recombinant, attenuated HIV” US Patent # 5585263 Issued 12/17/96.

Bray, M., Prasad, S. Dubay, J.W., Hunter, E. Jeang, K.T., Rekosh, D. and Hammar skjöld-M-L. (1994) A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. *Proc. Natl. Acad.Sci.(USA)* 91: 1256-1260.

Using the CTE as a component of our expression vectors has allowed us to, create a series of expression vectors that allows expression of HIV proteins in a Rev-independent fashion. The vectors were then used to create stable cell lines that expressed the proteins. One cell line in particular has proved extremely useful. It is called 2A.22. The cell line expressed HIV proteins (Gag-GagPol and Envelope) in a Rev-independent fashion. The creation of this cell line and its properties was described in the following publication: Srinivasakumar, N., Chazal, N., Helga-Maria, C., Prasad, S., Hammarskjöld, M.-L., and Rekosh, D. (1997) The effect of viral regulatory protein expression on gene delivery by human immunodeficiency virus type 1 vectors produced in stable packaging cell lines. *J. Virol* 71:5841-5848.

We are currently modifying 2A.22 by transfection with a vector that produces secreted alkaline phosphatase (SEAP). Since the SEAP to p24 ratio can readily be measured simply by assaying the medium, having such a modification will allow us, in a drug screening assay, to readily identify compounds that inhibit p24 production, but not the control SEAP gene.

One of the two necessary regulatory genes in the HIV genome, Rev initiates export of full length and partially processed HIV RNAs from the nucleus to the cytoplasm, a necessary event for HIV replication. Rev functions through the binding of RNA encoded Rev responsive element (RRE), an approximately 230 nucleotide sequence, followed by binding to cellular CRM1. This ribonuclear protein complex is then shuttled out of the nucleus using other cellular machinery in the nuclear pore.

RNA export elements are found in other RNA viruses. One example is the Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE). Similar to the RRE, the CTE is a small nucleotide sequence found on full length and partially processed MPMV RNAs. Circumventing the need for an MPMV analog of Rev, the CTE attaches directly to cellular machinery to initiate nuclear export. Experiments have shown that replacing the HIV RRE with a MPMV CTE leads to Rev-independent HIV-1 RNA export to the cytoplasm.

The cell line 5BD.1 was created by transfecting COS cells with the wild type HIV-1 structural and regulatory genes *gag*, *gagpol*, *rev*, and *env*. Each of these genes are necessary but not sufficient for producing infectious HIV virions. Non-infectious virions are produced

in 5BD.1 cells via the same pathways as in CD4<sup>+</sup> cells. Inhibition of Rev with a library compound would therefore have the same effect on viral production in 5BD.1 cells as in CD4<sup>+</sup> cells.

2A.22 was created by transfecting COS cells with modified HIV-1 structural genes gag, gagpol, and env. These genes were modified to replace the Rev binding site with the MPMV CTE. This cell line produces non-infectious virions in a Rev-independent manner. The Rev-independence of 2A.22 is useful as a negative control while testing library compounds. When both cell lines 5BD.1 and 2A.22 are grown and tested with the same compound under similar conditions, a reduction in viral production in 5BD.1 and not in 2A.22 indicates a potentially positive score for that compound as a Rev-specific inhibitor. Alternately, if a compound reduced p24 levels in both 5BD.1 and 2A.22, this could indicate a possible harmful interaction with cellular machinery and would rule out that specific compound from further studies.

There is clear evidence that Human Immunodeficiency Virus (HIV) is the cause of AIDS and that drugs that inhibit the replication and production of infectious HIV particles are efficacious in the treatment of AIDS. This disclosure describes 12 compounds that we have discovered that are very effective inhibitors of HIV particle formation. The compounds may act by inhibiting HIV Rev function, HIV assembly, HIV particle budding or some other part of the HIV life cycle. The compounds are therefore likely to form the chemical basis for new drugs that could be used for the treatment of AIDS.

The inhibitory compounds of the present invention were identified using the 5BD.1 cell line to screen for drugs that inhibit HIV particle formation without showing toxicity in a 5 day cell survival assay. The amount of HIV particles released by budding from the 5BD.1 cell line into the culture medium was measured using a simple and straightforward ELISA assay. 40,000 compounds were screened and 12 were selected as "hits" based on their ability to inhibit HIV particle formation without showing toxicity in a 5 day cell survival assay.

### **Brief Summary**

The present invention is directed to novel HIV inhibitory compounds and the use of those compounds to treat patients that are HIV positive.

**Brief Summary of the Drawings**

Fig. 1 parts A- I show the ELISA data readout from the primary screen of 40,000 compounds plotted as a percentage of the control. Compounds that gave an inhibition of HIV particle formation below 50% were chosen for further study. There are 192 compounds that give values below 50%.

Fig. 2 shows the chemical structures and names of the 12 compounds being disclosed.

Fig. 3 shows a three concentration dose response experiment for the compounds added to the cell line. Each compound is named with an identifier along the X-axis of the graph. The Y-axis shows the percent inhibition of HIV particle release into the medium. The different concentrations of compound utilized are represented by the different color bars as shown in the figure legend. Details of the assay are given in part E.

Fig. 4 shows a six concentration dose response curve of the same compounds. Details are the same as for Fig 3 except that six concentrations of compound were tested.

Figs. 5 and 6 show 2 day MTS toxicity assays for the compounds in 5 BD.1 cells, the same cell line used for the drug screening. A three concentration MTS toxicity assay is shown in Fig 5 and a six concentration assay is shown in Fig 6. The Y-axis represents the percentage of live cells after two days of treatment with the compounds. The same concentrations used in Fig 3 and 4 are used in this figure, as indicated by the different colors. Details of the assay are given in part E.

Fig. 7 shows a six concentration cell viability assay using the MT-4 T-cell line. The Y-axis shows the number of cells surviving after 5 days of incubation with each compound. The concentration of each compound used is indicated by the different colors. The data is expressed as the percentage of the starting number of cells. Details of this assay are given in part E.

**Detailed Description of Embodiments****Definitions**

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.



As used herein, the term “purified” and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term “purified” does not necessarily indicate that complete purity of the particular molecule has been achieved during the process. A “highly purified” compound as used herein refers to a compound that is greater than 90% pure.

As used herein, the term “pharmaceutically acceptable carrier” includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans.

As used herein, the term “treating” includes prophylaxis of the specific disorder or condition, or alleviation of the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms.

As used herein, the term “halogen” or “halo” includes bromo, chloro, fluoro, and iodo.

The term “haloalkyl” as used herein refers to an alkyl radical bearing at least one halogen substituent, for example, chloromethyl, fluoroethyl or trifluoromethyl and the like.

The term “C<sub>1</sub>-C<sub>n</sub> alkyl” wherein n is an integer, as used herein, represents a branched or linear alkyl group having from one to the specified number of carbon atoms. Typically C<sub>1</sub>-C<sub>6</sub> alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, hexyl and the like.

The term “C<sub>2</sub>-C<sub>n</sub> alkenyl” wherein n is an integer, as used herein, represents an olefinically unsaturated branched or linear group having from 2 to the specified number of carbon atoms and at least one double bond. Examples of such groups include, but are not limited to, 1-propenyl, 2-propenyl, 1,3-butadienyl, 1-butenyl, hexenyl, pentenyl, and the like.

The term “C<sub>2</sub>-C<sub>n</sub> alkynyl” wherein n is an integer refers to an unsaturated branched or linear group having from 2 to the specified number of carbon atoms and at least one triple bond. Examples of such groups include, but are not limited to, 1-propynyl, 2-propynyl, 1-butylnyl, 2-butylnyl, 1-pentylnyl, and the like.

The term “C<sub>3</sub>-C<sub>n</sub> cycloalkyl” wherein n = 8, represents cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl.

As used herein the term “aryl” refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings including, but not limited to, phenyl, benzyl, naphthyl, tetrahydronaphthyl, indanyl, indenyl, and the like.

The term (C<sub>5</sub>-C<sub>8</sub> alkyl)aryl refers to any aryl group which is attached to the parent moiety via the alkyl group.

The term “heterocyclic group” refers to a mono- or bicyclic carbocyclic ring system containing from one to three heteroatoms wherein the heteroatoms are selected from the group consisting of oxygen, sulfur, and nitrogen.

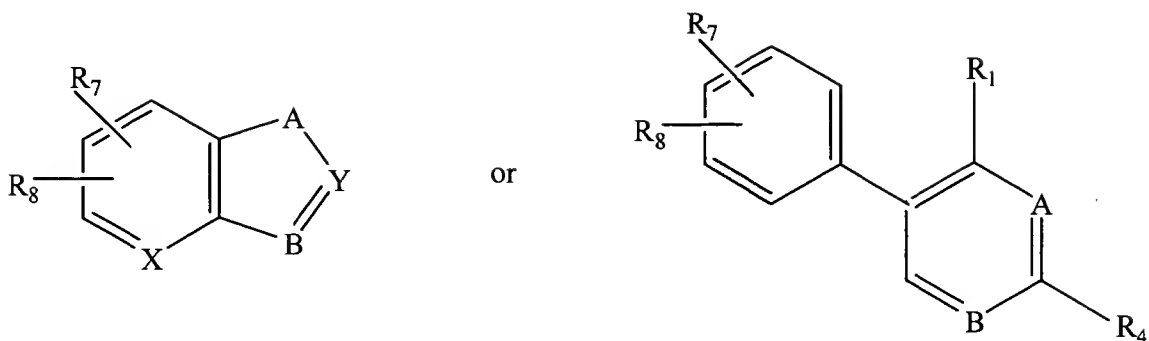
As used herein the term “heteroaryl” refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings containing from one to three heteroatoms and includes, but is not limited to, furyl, thienyl, pyridyl and the like.

The term “bicyclic” represents either an unsaturated or saturated stable 7- to 12-membered bridged or fused bicyclic carbon ring. The bicyclic ring may be attached at any carbon atom which affords a stable structure. The term includes, but is not limited to, naphthyl, dicyclohexyl, dicyclohexenyl, and the like.

The term “pharmaceutically-acceptable salt” refers to salts which retain the biological effectiveness and properties of the S1P analogs of the present invention and which are not biologically or otherwise undesirable. In many cases, the S1P analogs of the present invention are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.

## **Embodiments**

One aspect of the present invention is directed to novel compounds that inhibit the formation of HIV particles. In accordance with one embodiment an HIV inhibitor is provided wherein the compound has the general structure:



wherein

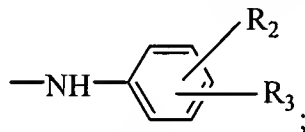
A is selected from the group consisting of N, CR<sub>1</sub>, and  $\text{---}\overset{\text{R}_1}{\underset{|}{\text{CHN}}}\text{---}$ ;

B is selected from the group consisting of N and S;

Y is selected from the group consisting of Se, CH and CR<sub>4</sub>;

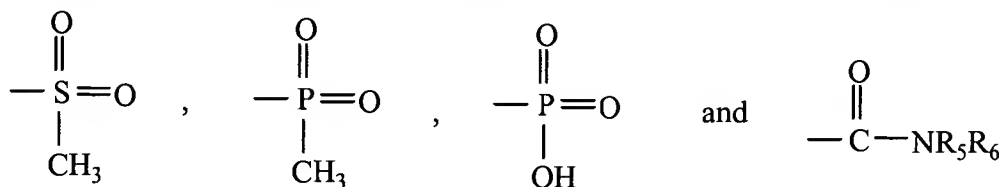
X is selected from the group consisting of CH and N;

R<sub>1</sub> is selected from the group consisting of H, NR<sub>5</sub>R<sub>6</sub> and



R<sub>2</sub> and R<sub>3</sub> are independently selected from the group consisting of H, halo, hydroxy and C<sub>1</sub>-C<sub>4</sub> alkyl;

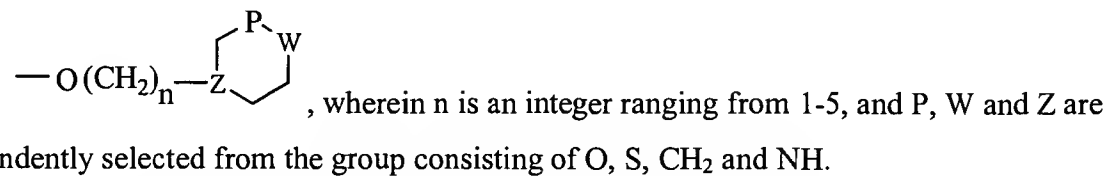
R<sub>4</sub> is selected from the group consisting of H, halo, hydroxy and C<sub>1</sub>-C<sub>4</sub> alkyl,



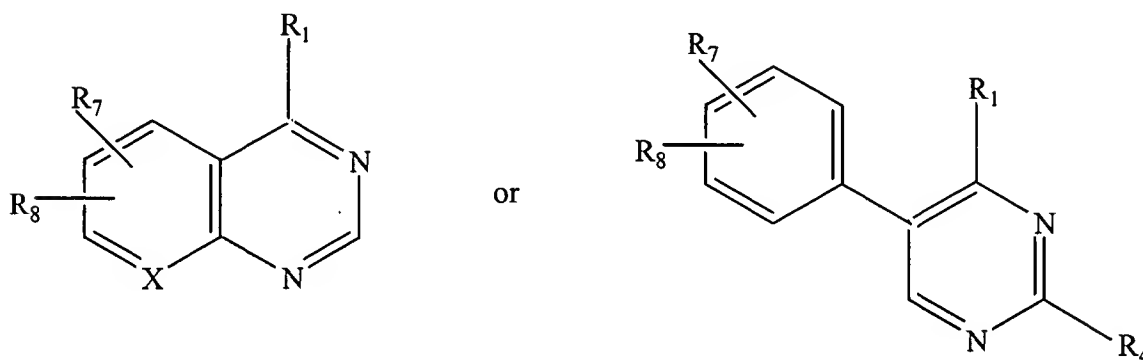
R<sub>5</sub> and R<sub>6</sub> are independently selected from the group consisting of H and C<sub>1</sub>-C<sub>4</sub> alkyl;

R<sub>7</sub> and R<sub>8</sub> are independently selected from the group consisting of H, halo, hydroxy, C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>1</sub>-C<sub>4</sub> alkoxy, -NHC(O)CH<sub>3</sub> and -O(C<sub>1</sub>-C<sub>4</sub> alkyl)(C<sub>5</sub>-C<sub>6</sub> heterocyclic) or R<sub>7</sub> and R<sub>8</sub> together with the atoms to which they are attached form an optionally substituted C<sub>5</sub>-C<sub>6</sub>

aryl, wherein the aryl ring is optionally substituted with halo, C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>1</sub>-C<sub>4</sub> alkoxy, C<sub>1</sub>-C<sub>4</sub> alkyl(C<sub>5</sub>-C<sub>6</sub> aryl) and -O(C<sub>1</sub>-C<sub>4</sub> alkyl)(C<sub>5</sub>-C<sub>6</sub> heterocyclic). In one embodiment Y is CR<sub>4</sub>, R<sub>7</sub> is H or C<sub>1</sub>-C<sub>4</sub> alkoxy, R<sub>8</sub> is halo or



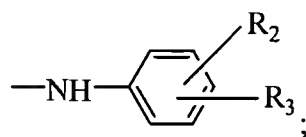
In another embodiment a compound is provided wherein the compound has the general structure:



wherein

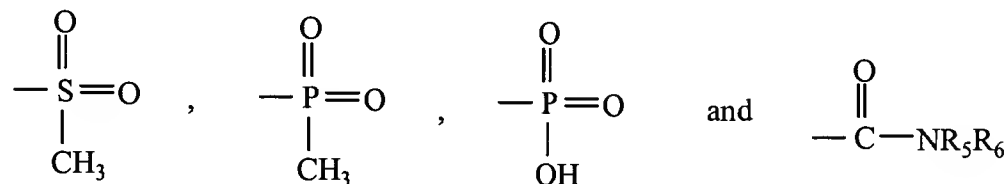
X is selected from the group consisting of CH and N;

R<sub>1</sub> is selected from the group consisting of H, NR<sub>5</sub>R<sub>6</sub> and



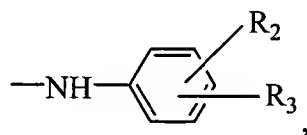
R<sub>2</sub> and R<sub>3</sub> are independently selected from the group consisting of H, halo, hydroxy and C<sub>1</sub>-C<sub>4</sub> alkyl;

R<sub>4</sub> is selected from the group consisting of H, halo, hydroxy and C<sub>1</sub>-C<sub>4</sub> alkyl,



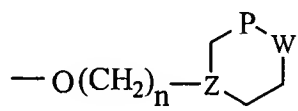
$R_5$  and  $R_6$  are independently selected from the group consisting of H and C<sub>1</sub>-C<sub>4</sub> alkyl;

$R_7$  and  $R_8$  are independently selected from the group consisting of H, halo, hydroxy, C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>1</sub>-C<sub>4</sub> alkoxy, -NHC(O)CH<sub>3</sub> and -O(C<sub>1</sub>-C<sub>4</sub> alkyl)(C<sub>5</sub>-C<sub>6</sub> heterocyclic) or  $R_7$  and  $R_8$  together with the atoms to which they are attached form an optionally substituted C<sub>5</sub>-C<sub>6</sub> aryl, wherein the aryl ring is optionally substituted with halo, C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>1</sub>-C<sub>4</sub> alkoxy, C<sub>1</sub>-C<sub>4</sub> alkyl(C<sub>5</sub>-C<sub>6</sub> aryl) and -O(C<sub>1</sub>-C<sub>4</sub> alkyl)(C<sub>5</sub>-C<sub>6</sub> heterocyclic). In one embodiment  $R_1$  is NR<sub>5</sub>R<sub>6</sub> or



$R_7$  is H or C<sub>1</sub>-C<sub>4</sub> alkoxy, and

$R_8$  is halo or



, wherein n is an integer ranging from 1-5, and P, W and Z are independently selected from the group consisting of O, S, CH<sub>2</sub> and NH. Another embodiment of the invention is directed to the compounds of Fig. 2.

The present invention is also directed to pharmaceutical compositions comprising the HIV inhibitory compounds of the present invention. More particularly, such compounds can be formulated as pharmaceutical compositions using standard pharmaceutically acceptable carriers, fillers, solubilizing agents and stabilizers known to those skilled in the art. Pharmaceutical compositions comprising the present compounds are administered to an individual in need thereof by any number of routes including, but not limited to, topical, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

In accordance with one embodiment, a method of treating HIV is provided. The method comprises the steps of administering a composition comprising an HIV inhibitory compound of the present invention to a patient in need thereof.

#### Example 1

Compounds were identified using a primary screening assay that involved the use of a cell line (5BD.1) that was continuously expressing HIV virus-like particles. To measure inhibition, supernatants containing HIV virus-like particles were obtained from the tissue culture assay described herein. The amount of HIV particles in the assay was then measured using the ELISA Assay that is also described below. The final ELISA data from the screening of approximately 40,000 compounds (commercially available from SPECS and BioSPECS, Rijswijk, The Netherlands) is shown in Figs 1A-I.

Compounds that reduced HIV particle formation by at least 50% as measured by these assays were screened further in the 3 and 6 point dose response assays and toxicity assays.

#### Primary Screening Assays

##### Tissue Culture Assay:

1. 5BD.1 cells were passaged in 2 T225 flasks in medium (IMDM/10% FCS/0.2 mg/ml HygromycinB/1.5 mg/ml G418/0.05 mg/ml gentamycin). Cells were trypsinized and harvested from 2 90% confluent flasks with  $28.4 \times 10^6$  cells recovered.
2. 4500 cells per well were plated into columns of tissue culture treated clear 384 well plates in 40  $\mu$ l per well of medium.
3. The plate was placed into the incubator for one hour.
4. Compounds were diluted from 2  $\mu$ l of 1 mM DMSO stocks in 384 well polypropylene plates by adding 38  $\mu$ l per well of medium.
5. 10  $\mu$ l of each diluted compound was transferred to the cell plates.
6. The plates were then incubated overnight for 16 hours.
7. In the morning of the next day the plates were aspirated on a plate washer.
8. 40  $\mu$ l of fresh medium was added to each well followed by 10  $\mu$ l of diluted as in step 5.
9. The plates were then incubated for 8 hours.
10. 25  $\mu$ l of supernatant per well from all wells was added to the plates coated and blocked below as described in step 14 below.

**p24 ELISA Assay**

11. Dilute primary antibody to 4 ug/ml in DPBS without calcium and magnesium, add 25 ul per well of a 384 well Maxisorp plate, incubate overnight at 4oC.
12. Aspirate coating solution, block for 30-60 minutes with 100 ul ELISA buffer (4 mg/ml BSA, 0.01% Tween20 in DPBS without calcium and magnesium).
13. Wash plates 2X.
14. Add 25ul of supernatant from step 10 above.
15. Add 10 ul of a 1:250 dilution of biotinylated secondary antibody in 25% lysis buffer/ELISA buffer.
16. Incubate overnight in the refrigerator.
17. Wash plates 3X.
18. Add 25 uL/well of a 1:10,000 dilution of detection SA-HRP. Incubate at room temp for 30 minutes.
19. Wash plates 3X. Add 25 uL/well of TMB substrate solution to all wells and develop for approximately 5 minutes until blue.
20. Stop the reaction with 25 uL/well 0.18M sulfuric acid.
21. Read plate at 450nm.

Compounds that gave 50% reduction in optical density as measured by this assay were identified and selected in this manner. The structure of each of twelve of these compounds being claimed in this disclosure is shown in Fig 2. (192 were selected originally).

**Dose Response Assays**

Each of the 192 compounds were then subjected to a 3 point dose response assay using the cell line. 33 compounds scored positive in the three dose response and were then subjected to a 6 point dose response. The data from these assays for the 12 compounds being disclosed here are given in figures 3 and 4. The two assays were performed as follows:

### **3 and 6 point Dose Response Assays**

#### **Tissue Culture**

1. 5BD.1 cells were carried in 2 T175 flasks in medium (IMDM/10% FCS/0.2 mg/ml HygromycinB/1.5 mg/ml G418/0.05 mg/ml gentamycin). Cells were trypsinized and harvested from a 90% confluent flask.
2. 20,000 cells per well were plated into rows A-G of 8 tissue culture treated clear 96-well plates in 135 ul per well of medium without G418 (assay medium). 135 ul of medium only was added to row H.
3. The plates were placed into the incubator for one hour.
4. Compounds were serially diluted 1:3 two times from 1 mM DMSO stocks in DMSO in 96 well polypropylene plates. 8 ul of the DMSO solutions were transferred to another plate and 72 ul of assay medium was added.
5. 15 ul of each diluted compound was transferred to the cell plates in duplicate with the high (10 uM final concentration in rows A-B, 3 uM concentration in rows C-D, 1 uM concentration in rows E-F and DMSO only in rows G-H).
6. The plates were then incubated overnight for 16 hours.
7. In the morning of the next day the medium was removed by hand from all wells.
8. 135 ul of fresh assay medium was added to each well followed by 15 ul of diluted compounds as described in step 5.
9. The plates were then incubated for 24 hours.
10. 50 ul of supernatant per well from all wells was harvested and added to the ELISA plates in step 15 below.

#### **p24 ELISA Assay**

11. Dilute primary antibody to 4 ug/ml in DPBS without calcium and magnesium, add 50 ul per well of a 96 well Maxisorp plate, incubate overnight at 4oC.
12. Aspirate coating solution, block for 30-60 minutes with 200 ul ELISA buffer (4 mg/ml BSA, 0.01% Tween20 in DPBS without calcium and magnesium).
13. Wash plates 2X.
14. Add 50 ul of the supernatants from step 10 above.



15. Add 15 ul of a 1:750 dilution of biotinylated secondary antibody in 40% lysis buffer/ELISA buffer.
16. Incubate 2 hours room temp with shaking.
17. Wash plates 3X.
18. Add 50 uL/well of a 1:10,000 dilution of detection SA-HRP. Incubate at room temperature with shaking for 30 minutes.
19. Wash plates 3X. Add 50 uL/well of TMB substrate solution to all wells and develop for approximately 15 minutes until blue.
20. Stop the reaction with 50 uL/well 0.18M sulfuric acid.
21. Read plate at 450nm.

#### **Toxicity Assays:**

##### **MTS-Assay**

MTS-based toxicity assays were performed in parallel to the 3 and 6 point dose response assays. The assay uses MTS a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(b)] and an electron coupling reagent (phenazine ethosulfate; PES) and was performed according to the directions of its manufacturer Promega, Madison Wisconsin. (see attached protocol Technical Bulletin #245 from Promega). The MTS assay data for the 12 compounds disclosed here after two days of incubation with 5BD.1 cells are shown in figures 5 and 6 and after incubation for 5 days with MT-4 T cells are shown in Figure 7.

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Figure 1A

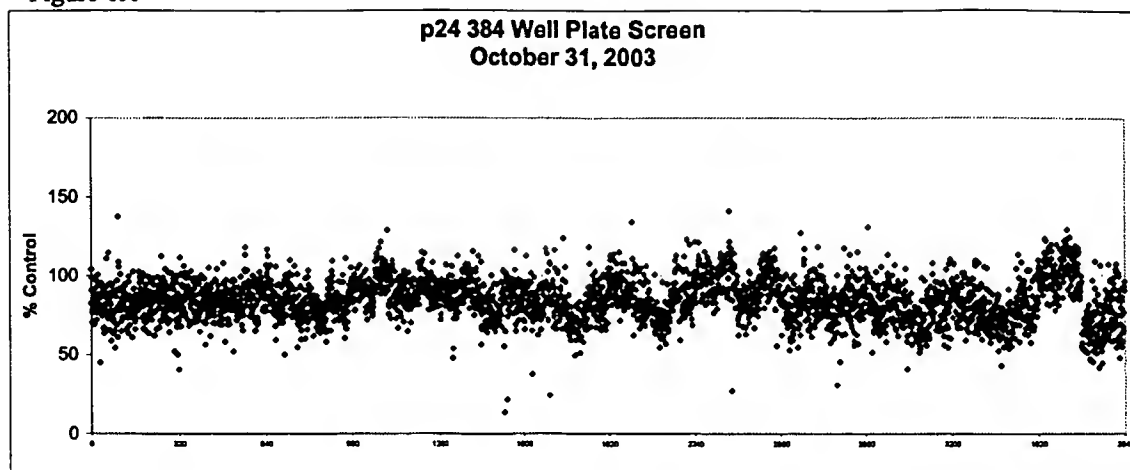


Figure1B

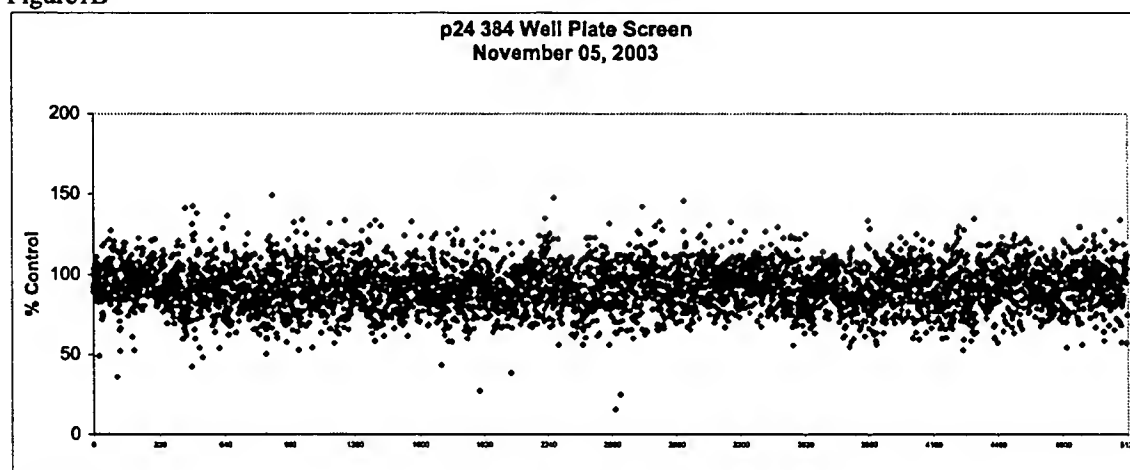


Figure1C

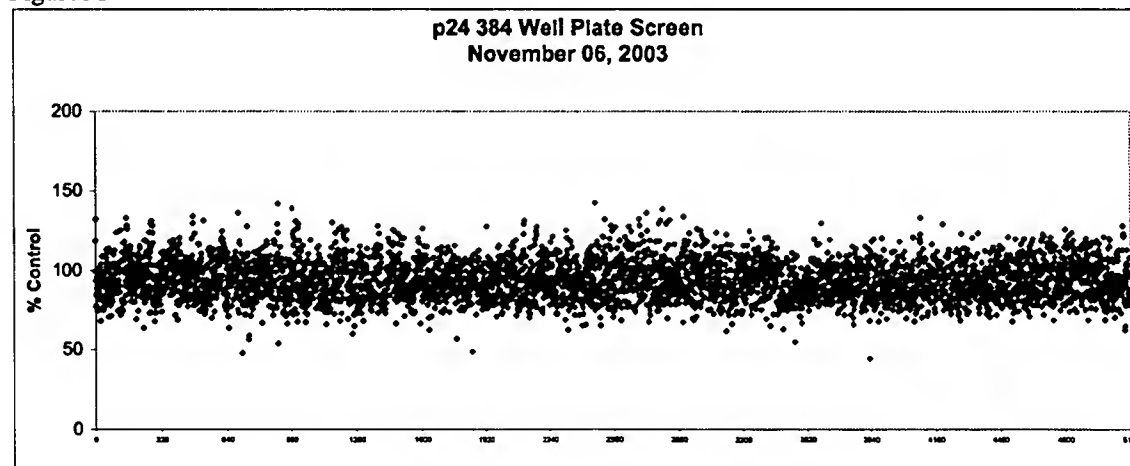


Figure1D

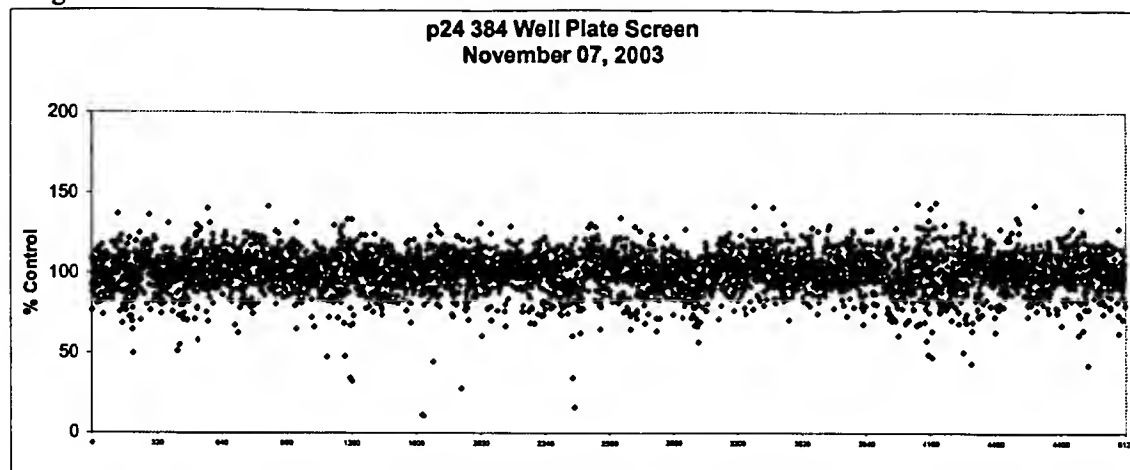


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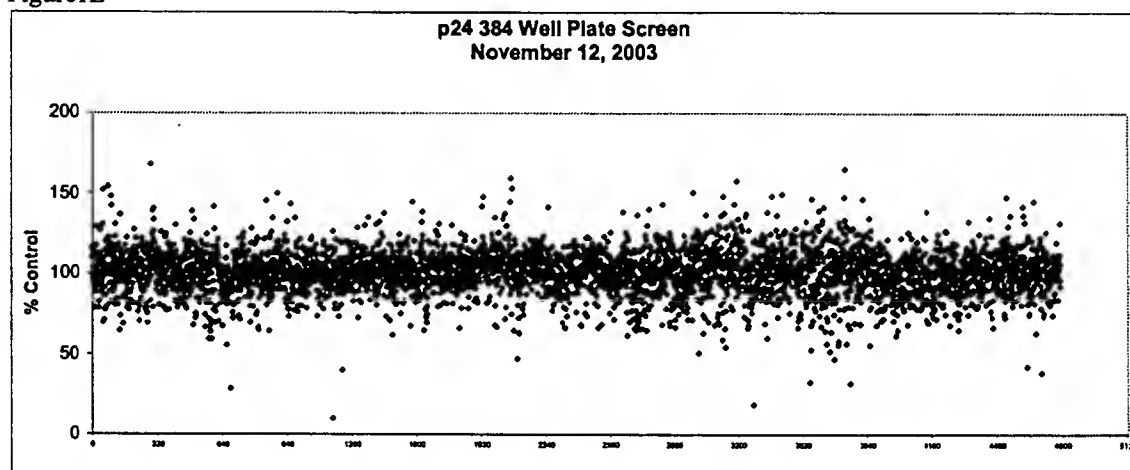


Figure1F

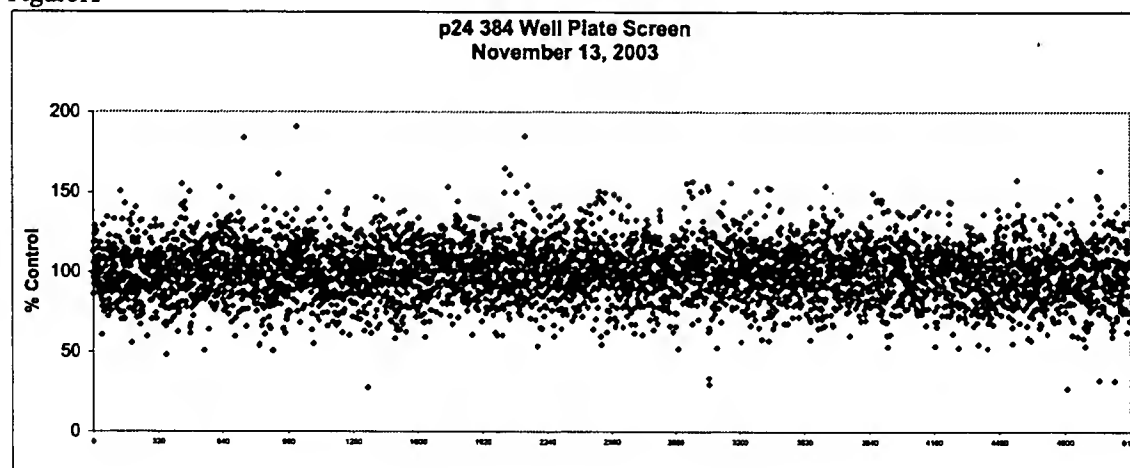


Figure 1G

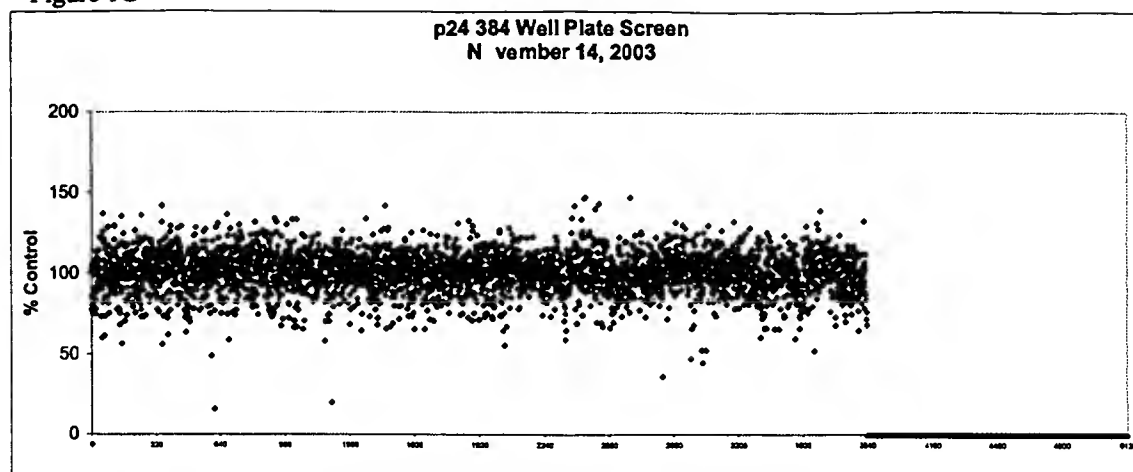


Figure 1H

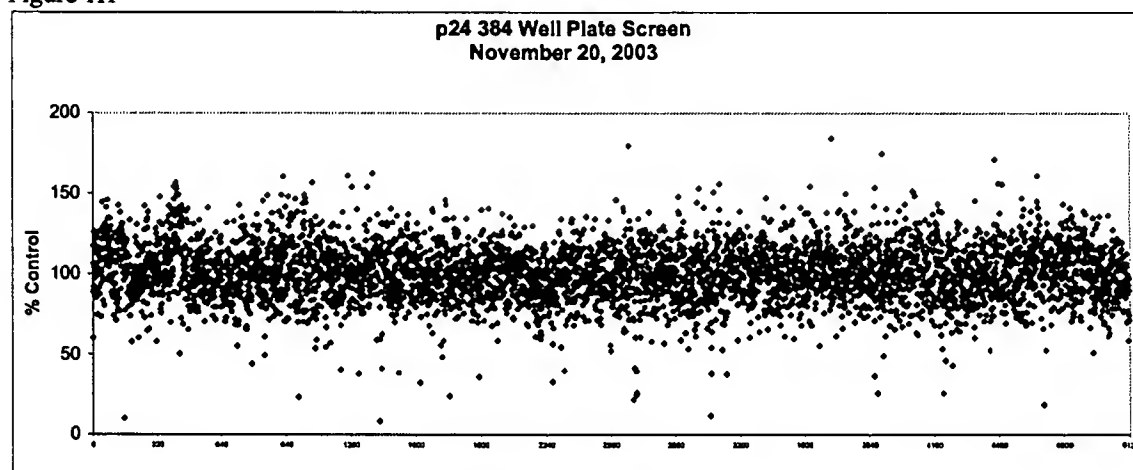


Figure 1I

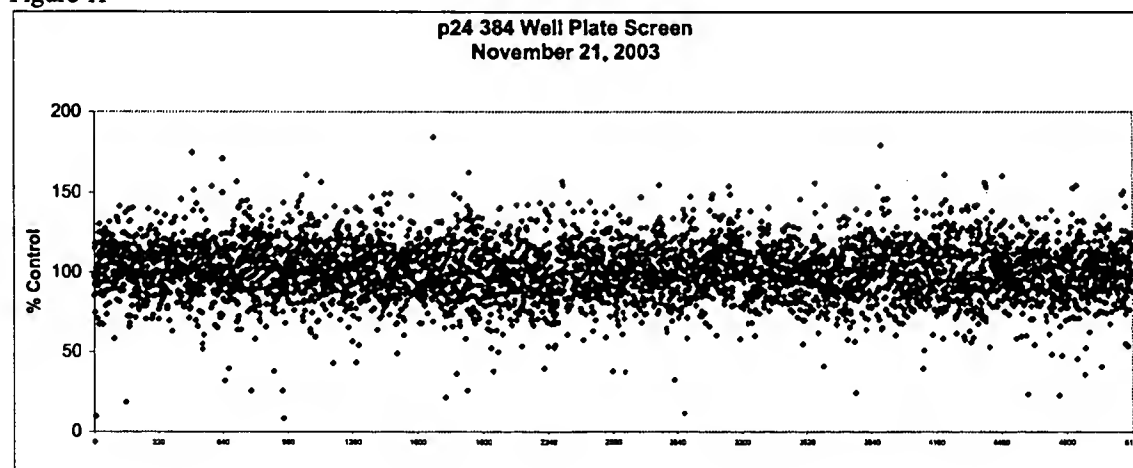
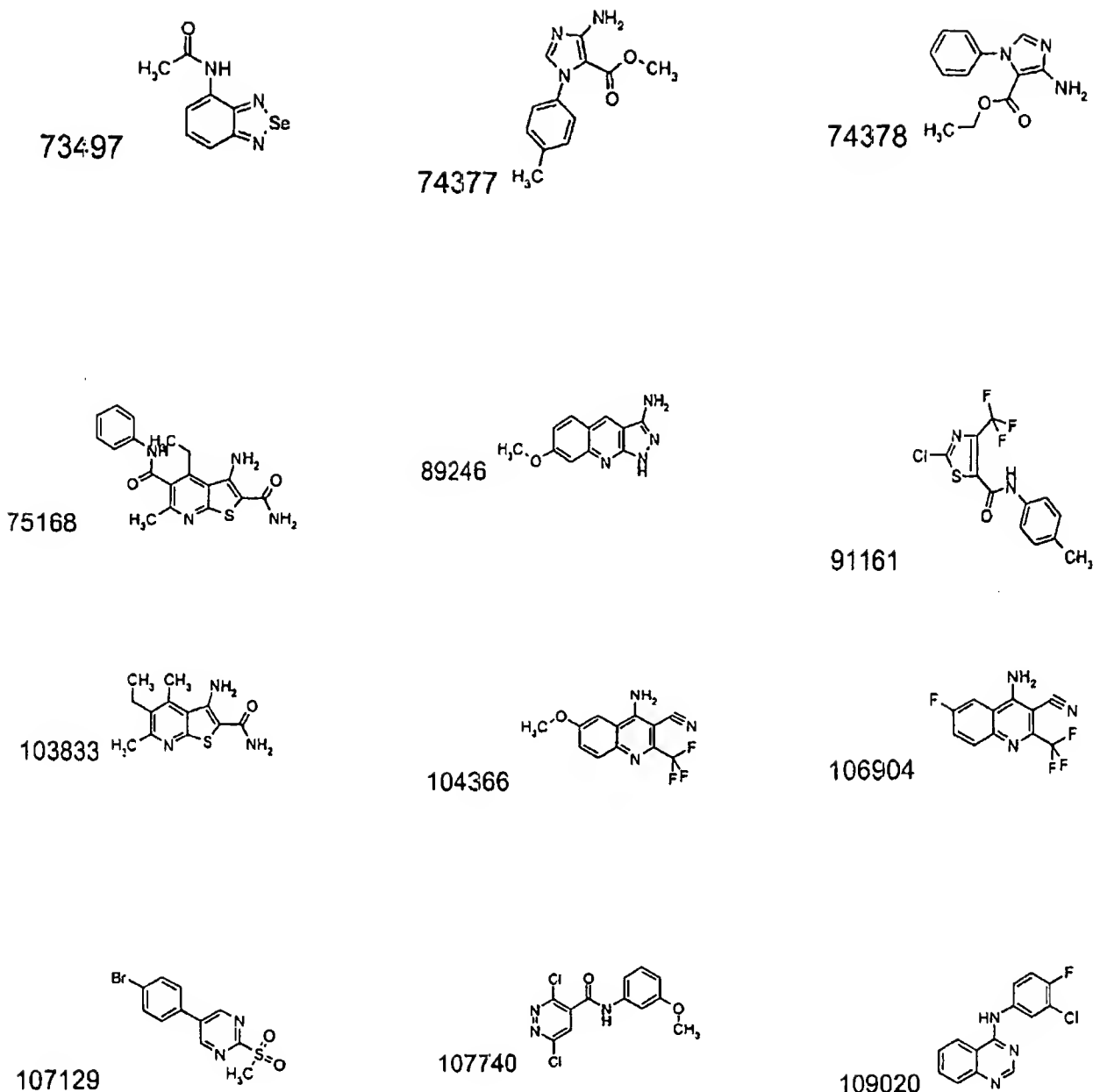
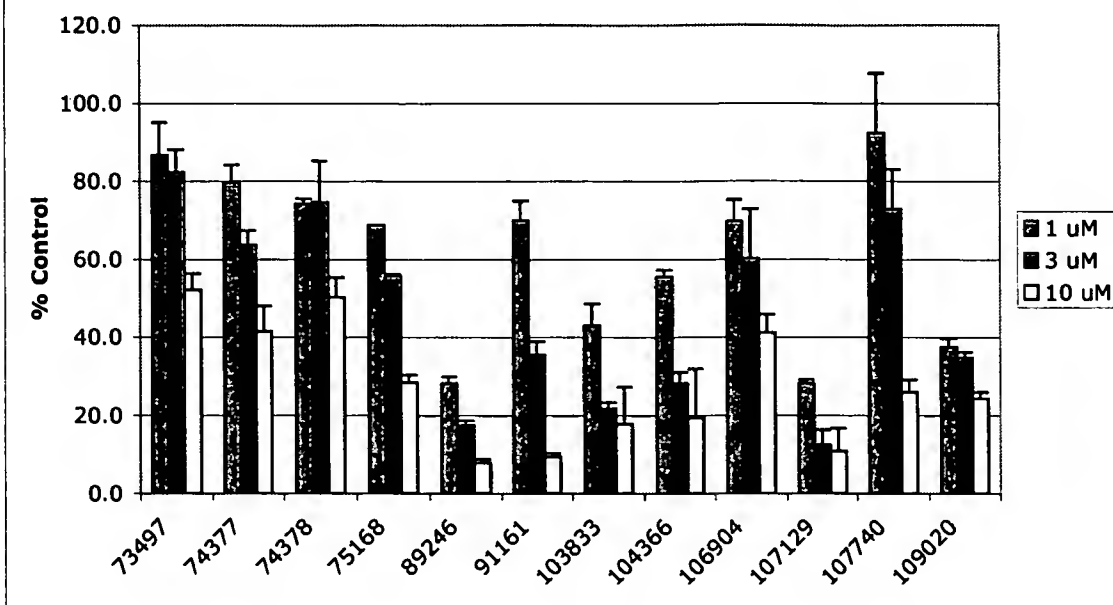


Figure 2

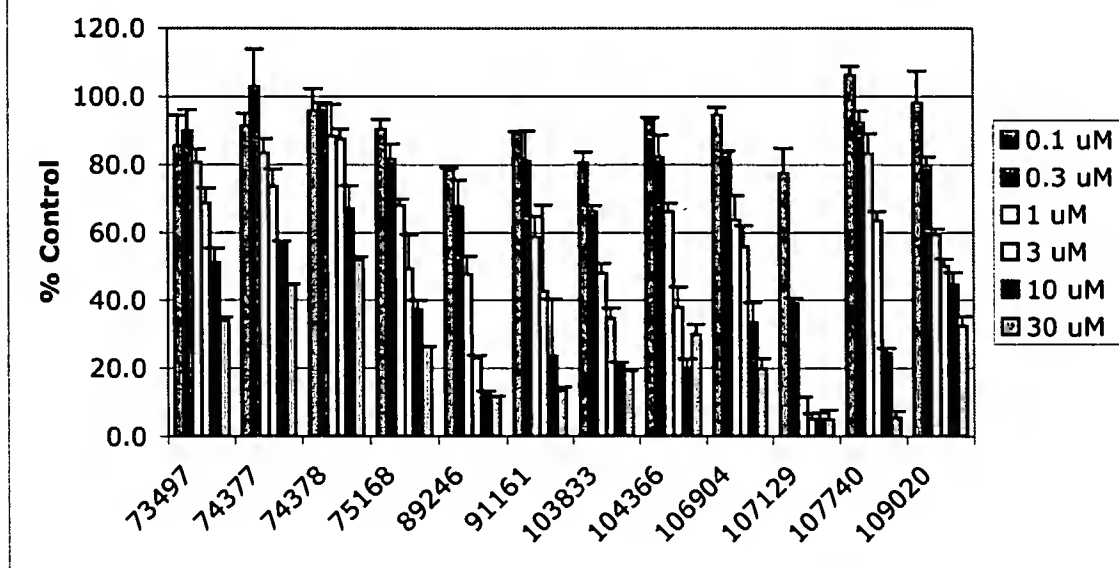


ID-Number	Name
74377	methyl 4-amino-1-(4-methylphenyl)-1H-imidazole-5-carboxylate
74378	ethyl 4-amino-1-phenyl-1H-imidazole-5-carboxylate
73497	N-(2,1,3-benzoselenadiazol-4-yl)acetamide
75168	3-amino-4-ethyl-6-methyl-N-5-phenylthieno[2,3-b]pyridine-2,5-dicarboxamide
89246	7-methoxy-1H-pyrazolo[3,4-b]quinolin-3-ylamine
91161	2-chloro-N-(4-methylphenyl)-4-(trifluoromethyl)-1,3-thiazole-5-carboxamide
103833	3-amino-5-ethyl-4,6-dimethylthieno[2,3-b]pyridine-2-carboxamide
104366	4-amino-6-methoxy-2-(trifluoromethyl)-3-quinolinecarbonitrile
106904	4-amino-6-fluoro-2-(trifluoromethyl)-3-quinolinecarbonitrile
107129	5-(4-aminophenyl)-2-pyrimidinethiol
107740	3,6-dichloro-N-(3-methoxyphenyl)-4-pyridazinecarboxamide
109020	N-(3-chloro-4-fluorophenyl)-N-(4-quinazolinyl)amine

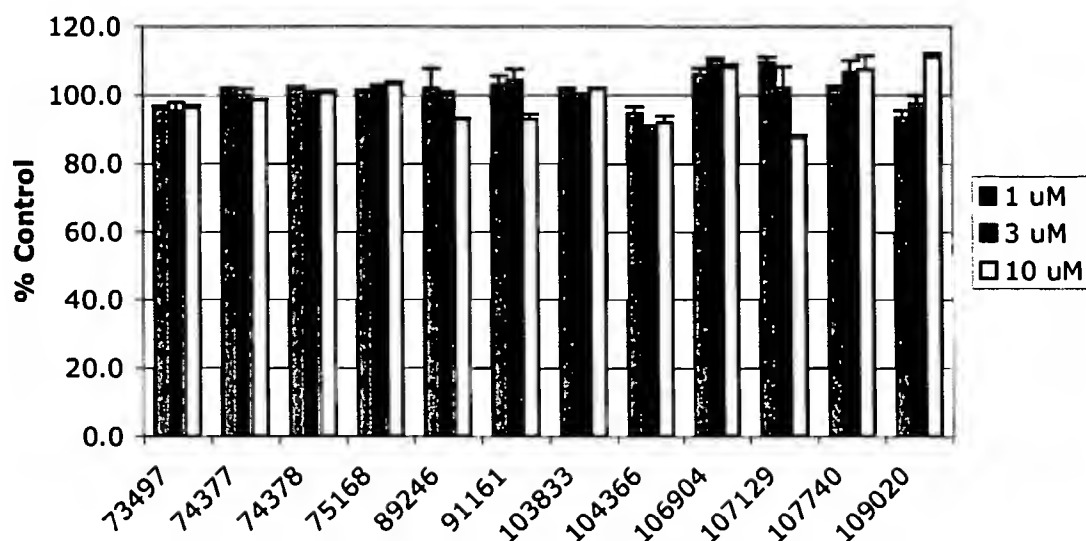
**Fig. 3: 3-P int D se Response f P24**



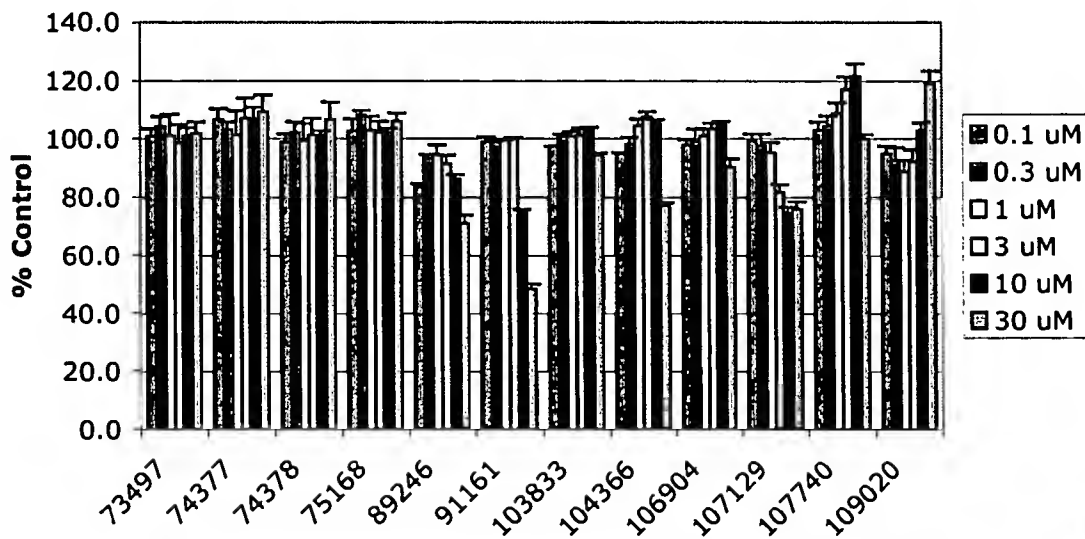
**Fig. 4: 6-Point Dose Response of P24**



**Fig. 5: 3-Point Viability (MTS Toxicity)**



**Fig. 6: 6-Point Viability (MTS Toxicity)**





**Fig. 7: 6-P int MT4 Viability**

